Aggregation-promoting factors in neurodegenerative diseases

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CHAPTER VII

Discussion and perspectives

Many neurodegenerative diseases are characterized by clinicopathological features that affect motor capacity and cognition in affected patients. Examples of such diseases are Alzheimer's, Parkinson's, polyglutamine diseases and amyotrophic lateral sclerosis (ALS). Unfortunately, no cure is currently available and the risk of developing these diseases increases with aging. Interestingly, a hallmark of post-mortem biopsies from the brains of affected patients is the presence of protein aggregates in neurons. While specific aggregation-prone proteins have been identified for each neurodegenerative disease, their contribution to disease remains unresolved. More specifically, we do not know what triggers protein aggregation and how this is mechanistically linked to pathogenesis. What we do know is that protein homeostasis is affected in these diseases, and understanding how it is altered may help us not only to identify the cellular processes involved but also – in the long term – to target them for potential disease-modifying treatments.

The aim of the work presented in this thesis was to dissect the cellular and molecular aspects of protein aggregation, in order to find mechanisms that help to explain how protein homeostasis correlates with pathogenesis. A main goal was to discover genetic regulators of protein aggregation in an unbiased and hypothesis-free manner, with the expectation of identifying new regulators and therefore new mechanisms in protein aggregation.

We were successful in identifying one such new regulator in the form of MOAG-2/LIR-3. **Chapter IV** describes the identification and characterization of this modifier of aggregation in a *C. elegans* model of polyglutamine diseases. We discovered that MOAG-2/LIR-3 is an RNA Polymerase (Pol) III-associated transcriptional regulator that drives polyglutamine aggregation. In this chapter we propose a mechanism whereby aggregation-prone proteins recruit normally functioning cellular proteins to promote their own aggregation. Despite this important finding, this study leaves several questions unanswered – these are discussed below, together with suggestions for directions of future research in the context of MOAG-2/LIR-3 and the link between the non-coding genome and protein aggregation.

1. Aggregation-prone proteins affect the non-coding genome

One surprising finding from this work was that in our model the presence of polyglutamine expansion proteins induced the downregulation of small non-coding RNAs (ncRNAs) – specifically snRNAs, snoRNAs and tRNAs – demonstrating that the non-coding genome is also affected by the presence of aggregation-prone proteins. It is still unknown how this downregulation occurs and we propose two different possibilities that could explain this observation. The first possibility is that misfolded, aggregation-prone proteins directly sequester these small ncRNAs into aggregates, thereby reducing their abundance in the cell. The second possibility is that aggregation-prone proteins sequester MOAG-2/LIR-3, subsequently inhibiting it from driving small ncRNA transcription. To experimentally address these hypotheses, it would be necessary to isolate the polyglutamine aggregates and detect the presence of small ncRNAs (e.g. qPCR) or MOAG-2/LIR-3 (e.g. co-immunoprecipitation).

The next unresolved question is what biological consequences the downregulation of small ncRNAs might have. For example, the reduction of one type of small ncRNAs in the nucleus – the U1 snRNAs – affects the splicing of premature mRNA [1, 2]. In our case, however, downregulation of snRNAs did not affect the splicing of premature mRNA (data not shown). Our observations so far point to the notion that it is possible to retain functional splicing events despite lower levels of splicing factors. The downregulation of another type of small ncRNA – the tRNAs – may also have biological consequences, and it will be interesting to investigate how tRNA function is affected, if at all (e.g. is translation efficiency maintained? does the proteome change?).

Finally, it will also be worth assessing whether the downregulation of small ncRNAs is cell type-specific (e.g. do polyglutamine-expressing cells have a distinct ncRNA repertoire?) and whether altered ncRNA homeostasis is specific to polyglutamine expansion proteins or shared by other aggregation-prone proteins (e.g. alpha-synuclein, amyloid-beta).

2. Is MOAG-2/LIR-3 related to calcium-regulated transcription factors?

Once we discovered that MOAG-2/LIR-3 was a transcription factor we were interested to know whether it shared homology with other mammalian transcription factors. Protein sequence comparisons revealed that MOAG-2/LIR-3 is predominantly conserved among the *Caenorhabditis* genus. It does, however, share 27% homology with the human calcineurin B homologous protein 1 (CHP1) and 37% homology with the human zinc finger protein 64 (ZNF64). The presence of these two functional motifs – a calcium-binding domain and a zinc finger – is typical of human calcium-regulated transcription factors, such as the cAMP-responsive element-binding protein (CREB)-binding protein (CBP), the downstream regulatory element antagonist modulator (DREAM) and FOXO3A [3]. The combination of these two domains – calcium binding and DNA binding – poses the interesting possibility that MOAG-2/LIR-3 may be a calcium-dependent transcription factor that regulates expression of small ncRNAs.

3. What are the proteins that interact with MOAG-2/LIR-3?

We can learn more about MOAG-2/LIR-3 function by identifying the types of proteins with which it interacts or forms complexes. We have shown that MOAG-2/LIR-3 binds to the same promoters as the RNA Pol III complex. We have also shown that inactivation of MOAG-2/LIR-3 reduces the levels of transcribed small ncRNAs, suggesting that MOAG-2/LIR-3 cooperates with the RNA Pol III complex to promote small ncRNA transcription. The question of whether MOAG-2/LIR-3 is physically part of the RNA Pol III complex could be answered by using purified MOAG-2/LIR-3 in co-immunoprecipitation experiments, followed by mass spectrometry. This approach would also enable us to identify other co-immunoprecipitated proteins and establish a protein interaction network for MOAG-2/LIR-3, which would tell us more about MOAG-2/LIR-3 function and whether it cooperates with other co-factors to regulate the expression of small ncRNA genes.

4. Could MOAG-2/LIR-3 be an RNA-binding protein?

The C2H2 zinc fingers of MOAG-2/LIR-3 are structurally homologous to the canonical C2H2 zinc fingers of the transcription factor IIIA (TFIIIA) in *Xenopus laevis* [4]. TFIIIA is a promoter-bound recruitment factor that recruits TFIIIB which, in turn, recruits Pol III to the promoters of genes encoding of 5S rRNA [5]. The binding of TFIIIA to the promoter takes place through physical binding of zinc fingers 4-9 to two specific regions: Box A and the intermediate element [6]. In addition to binding to 5S promoters, TFIIIA also binds to 5S RNA itself through zinc fingers 4-7 [7-9]. The fact that we found MOAG-2/LIR-3 to be frequently bound to 5S promoters suggests that it may also act as a recruitment factor, recruiting other components of the RNA Pol III complex to Box A and Box B. Furthermore, the resemblance of motifs within its structure to the zinc fingers of TFIIIA opens up the possibility that MOAG-2/LIR-3 might bind directly to rRNA or to any other type of RNA, a prospect that will have to be assessed in future studies.

5. Do aggregation-prone proteins alter binding of MOAG-2/LIR-3 to its transcriptional targets?

We have shown that MOAG-2/LIR-3 binds to the promoters of snRNA, snoRNA and tRNA genes to promote their transcription. One open question stemming from this work is how the presence of aggregation-prone proteins modifies the binding of MOAG-2/LIR-3 to its transcriptional targets. We propose that polyglutamine sequesters MOAG-2/LIR-3 or modifies its localization, thereby preventing MOAG-2/LIR-3 binding to its DNA targets such that it can no longer transcribe small ncRNAs with the same efficiency as that in wild type animals. This could explain why we observe lower levels of small ncRNAs in the presence of aggregation-prone proteins. Indeed, it would be interesting to verify whether MOAG-2/LIR-3 can still retain its function as a transcriptional regulator in polyglutamine-expressing animals, a hypothesis which will require testing in additional ChIP seq experiments.

The identification and characterization of MOAG-2/LIR-3 in *C. elegans* offers a major contribution to our understanding of the aggregation pathway in neurodegenerative diseases, and in particular how benign cellular proteins can convert their function to an aggregation-promoting factor. A second major

step focused on the effects of protein aggregation at the transcriptional level. In **Chapter V**, we showed that the presence of polyglutamine-expansion proteins induced the expression of stress response genes involved in: 1) oxidative stress; 2) the ER-associated unfolded protein response (UPR); and 3) the innate immune response. Oxidative stress results from the imbalance between the production and clearance of reactive oxygen species (ROS), which can cause damage to DNA, proteins and other cellular components (reviewed in [10]). Our results come in line with previous published work implicating oxidative stress in Huntington's disease [11, 12]. It would be interesting to further explore this correlation and determine what specific cellular events lead to oxidative stress (e.g. mitochondrial dysfunction? overproduction of ROS? insufficient antioxidant production?).

If unfolded proteins accumulate in the ER, the UPR is responsible for restoring protein homeostasis by fine-tuning protein-folding load with protein-folding capacity (reviewed in [13]). Previous published work showed that the UPR contributes to neuroprotection in a *C. elegans* model expressing alpha-synuclein in the dopaminergic neurons [14]. Although polyglutamine expansion proteins are not expected to enter the secretory pathway, it is still reasonable to consider that their accumulation at the ER (perhaps immediately after protein synthesis) would be sufficient to trigger the UPR as protective response to aggregation-prone proteins.

A surprising finding was the involvement of the innate immune response to polyglutamine expansion proteins. Several genes that encode antimicrobial peptides were upregulated in our study, suggesting that aggregation-prone proteins can also elicit a systemic effect in the organism. It is possible that aggregation-prone proteins are perceived by the organism as a pathogen and thereby elicit the immune system to counteract their toxicity. Indeed, the involvement of the cellular stress responses described here show that the organism takes advantage of distinct cellular defense mechanisms at its disposal to combat the pathogenic presence of aggregation-prone proteins. Following this line of thought, it would be interesting to investigate whether there is a cross talk between these cellular responses to aggregation-prone proteins.

Lastly, we saw that the effects of aggregation-prone proteins go beyond cellular stress responses and can also delay *C. elegans* development. Polyglutamine diseases are not known to be associated with impaired development, therefore suggesting that the developmental delay observed in our polyglutamine model may be specific to nematodes.

A final major step in our understanding of protein aggregation is well under way as a result of our work with MOAG-4 and its mammalian orthologs, SERF1A and SERF2. Below we discuss the contributions made by our study on SERF2 in the brains of mice (**Chapter VI**) and discuss plans for future experiments in mouse models of Alzheimer's disease.

The aggregation-prone protein most commonly associated with Alzheimer's disease (AD) is amyloid-beta (reviewed in [15, 16]). We and others have previously shown that aggregation of amyloid-beta is promoted both *in vitro* and *in vivo* by MOAG-4/SERF [17, 18]. In a *C. elegans* model of amyloid-beta, the transgene is expressed intracellularly in the body wall muscle, where it accumulates and contributes to progressive paralysis in the worm [19]. We have shown that deletion of MOAG-4 ameliorates this paralysis phenotype by reducing the amount of seeding-competent amyloid-beta [17]. In addition, our studies in human cell models have shown that the function of MOAG-4 is conserved in two mammalian orthologs, SERF1A and SERF2 [17]. This led us to hypothesize that SERF proteins could be driving amyloid-beta aggregation in the AD brain. To test this hypothesis, we generated a *Serf2* knockout mouse and crossed it with an AD mouse model. In **Chapter VI**, we discovered that a full body knockout of *Serf2* results in embryonic lethality with incomplete penetrance. Since this does not allow us to study the role of *Serf2* in adult mice, we generated brain-specific *Serf2* knockout mice instead. As a next step in this project, we plan to mate these animals with AD mouse models.

The aim of generating a brain-specific *Serf2* knockout mouse in an AD background is to find out whether the function of MOAG-4/SERF in proteotoxicity is conserved from *C. elegans* to mammals. Specifically, we want to know whether removing SERF from the brain in AD mouse models suppresses amyloid aggregation and neurotoxicity. We also want to find out whether

changes in proteotoxicity are accompanied by changes in motor and cognitive functions. The experimental design is depicted in Figure 1. Brain-specific *Serf2* knockout mice – *Sox1 Cre*$^{+}$ *Serf2*$^{-/-}$ – will be mated with two distinct AD mouse models. The first strategy involves mating the *Sox1 Cre*$^{+}$ *Serf2*$^{-/-}$ mice with the APPPS1 mouse model [20]. In this model, the human amyloid precursor protein (APP) harboring the K595N/M596L "Swedish" mutation (otherwise known as K670N/M671L) and the human presenelin 1 (PS1) protein harboring the L166P mutation are co-expressed under the Thy1 promoter, which directs transgene expression to the postnatal brain [20]. Both mutations are associated with early onset AD in humans. In this model, amyloid-beta deposition is seen as early as six weeks of age, with cognitive impairment appearing at 7 months of age [20, 21]. From the mating between the *Sox1 Cre*$^{+}$ *Serf2*$^{-/-}$ mice and the APPPS1 mice, we expect to obtain progeny in Mendelian proportions with the following characteristics: 1) wild type for all transgenes; 2) transgenic for APPPS1; 3) wild type lacking SERF2 in the brain; and 4) transgenic for APPPS1 and lacking SERF2 in the brain (Figure 1). Here, we will focus predominantly on brain histology and immunohistochemistry to study the effect of a lack of *Serf2* in the brain on amyloid-beta deposition (Figure 1).

In the second strategy, we will mate *Sox1 Cre*$^{+}$ *Serf2*$^{-/-}$ mice with the APPswe/PSEN1dE9 mouse model, from which we also expect to obtain all four desired phenotypes in Mendelian proportions (Figure 1) [22]. In this model of AD, a chimera of mouse and human APP harboring the K595N/M596L "Swedish" mutation (APPswe) is co-expressed with the exon-9-deleted (delta E9) variant of human mutated PS1 protein (PSEN1dE9), the latter also associated with early onset AD [22]. The expression of both transgenes is under the mouse prion promoter, which restricts their expression to the neurons of the central nervous system [22]. In APPswe/PSEN1dE9 mice, amyloid-beta plaques typically appear at 4-6 months of age, and plaque accumulation progresses up to 12 months of age, when cognitive deficits also start to appear [23, 24]. We will subject these mice to a battery of behavioral tests to assess spatial memory reference (e.g. Morris water maze), exploration and anxiety (e.g. open field) and motor capacity (e.g. grip strength) (Figure 1). In parallel, we will perform histological and immunohistochemical assays to assess amyloid deposition and neuronal toxicity. Since AD is a progressive disorder, we want to learn how aggregation

formation, neurotoxicity and cognition are affected during aging. For this reason, we will include both 3 to 4 and 6 to 7-month old animals in our analysis. In all, we hope to uncover the function of *Serf2* in the brain and determine whether it acts as a genetic modifier of proteotoxicity and cognition in AD. Results from these studies will provide insight into how the brain copes with protein aggregation during aging and whether this is accompanied by changes in behavior. Ultimately, this study may reveal *Serf2* to be a potential therapeutic target for modulation of neurodegeneration.

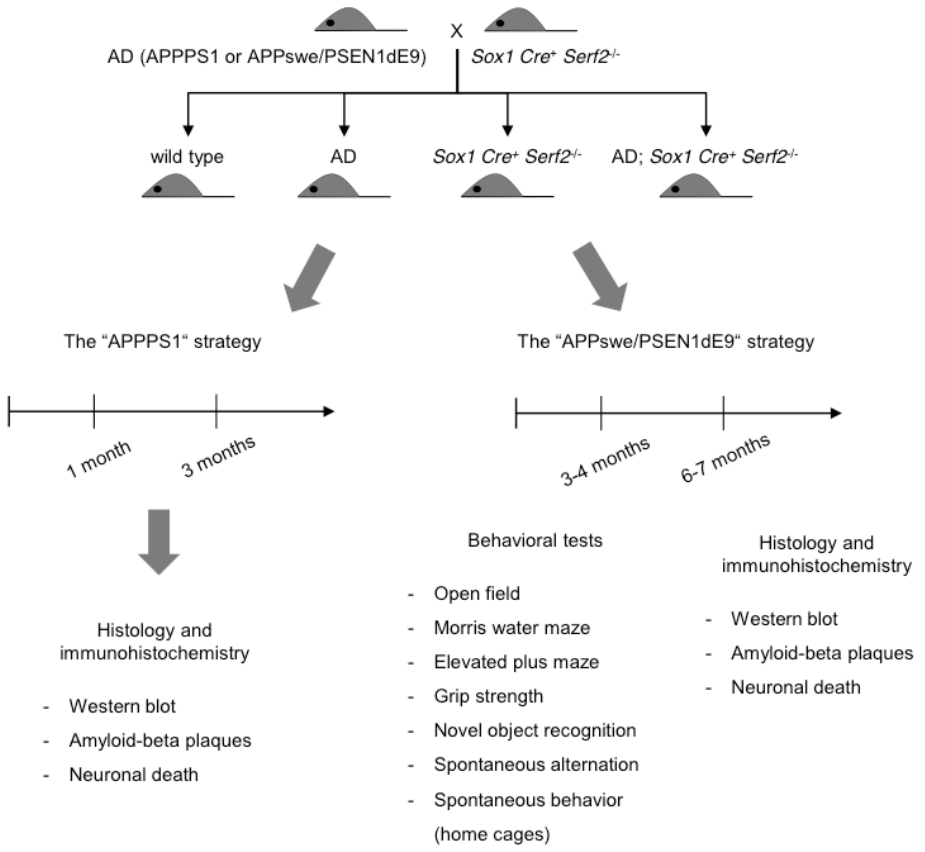

Figure 1. Diagram representing the generation of a brain-specific *Serf2* knockout mouse (*Sox1 Cre*$^{+}$ *Serf2*$^{-/-}$) in two different AD mouse models. In the "APPPS1" strategy, the brain-specific *Serf2* knockout mice will be mated with the APPPS1 mice and focus on histological and immunohistochemical analyses at both 1 and 3 months of age. In the "APPswe/PSEN1dE9" strategy, the brain-specific *Serf2* knockout mice will be mated with the APPswe/PSEN1dE9 mice. A set of behavioral tests will be performed parallel to histological and immunohistochemical analyses to 3 to 4 and 6 to 7-month old mice.

Outlook

In this thesis, we have demonstrated that aggregation-prone proteins influence ncRNA homeostasis, thereby introducing the concept that aggregation-prone proteins have effects that go beyond protein homeostasis. This demonstrates that it may be worth examining how the non-coding genome is altered in neurodegenerative diseases. We have also identified a new aggregation-promoting factor and hope to have uncovered yet another mechanism exemplifying how protein homeostasis can go awry during pathogenesis. Finally, we have provided a starting point for translational research, in which we intend to explore the possibility of a genetic modifier – discovered in *C. elegans* and conserved throughout evolution – being a potential therapeutic target for neurodegenerative diseases.

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